

The University of Chicago

STUDIES ON DISSOCIATION OF CER-
TAIN PARATYPHOID BACILLI

THE RÔLE OF VARIANTS IN THE PRECIPITATION OF
CALCIUM SULPHITE

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIO-
LOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

By
MARY E. CALDWELL

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THE RÔLE OF VARIANTS IN THE PRECIPITATION OF CALCIUM SULPHITE

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The University of Chicago and the University of Arizona

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Although bacterial variation has attracted attention since the early days of bacteriological investigations, interest in this field in recent years has been greatly stimulated by the work of Arkwright (1921), Schütze (1921), de Kruif (1921) and others. Since this literature has been comprehensively reviewed by Hadley (1927, 1931a) an attempt will be made to avoid repetition. This paper deals with what are thought to be variant cells which have the ability to precipitate calcium sulphite in a suitable environment; these have arisen under conditions conducive to dissociation and, as far as the author is aware, have not been reported. In some respects they seem to be related to the familiar variants of usual colony forms of certain bacteria under enforced dissociation.

In the course of bacteriophage studies, sealed filtrates of *Salmonella Schottmülleri* frequently became opalescent or cloudy. Such observations have been reported by Hauduroy (1927), d'Herelle et al. (1930) and others on certain bacterial filtrates. Agar plates streaked from these *S. Schottmülleri* filtrates gave rise to very small, opaque bodies (fig. 1) which were superimposed upon and at times present in the medium surrounding ordinary colonies. These will be referred to as thiosomes which, due to their opacity, were black by transmitted light, white by reflected light, showed extinction with crossed nicols and were iridescent with polarized light. Apparently associated with the thiosomes were more typically daughter-colony-like structures herein re-

ferred to as atypical thiosomes (figs. 4 and 6). These were devoid of blackness, varied in their degrees of translucency, were not anisotropic and never occurred outside the colony. Upon a synthetic medium and upon ordinary fresh veal infusion medium only atypical thiosomes appeared in the colonies.

Under different conditions, more or less obscure, thiosomes exhibited variations as to size, shape, occurrence, relationship with R (rough) and S (smooth) colonies and reactions to different media. For example, in size, some resembled the finest of soot particles, while others were regarded as small, as intermediate or as large, when viewed with a colony microscope ($\times 40$), i.e., they varied in diameter from 0.005 to 0.015 mm. Those of the soot-like type were sometimes heaped upon a colony to such an extent that the margin of the latter was obscured; again, they were confined to a definite area on the colony, for instance, forming a submarginal zone. Within this zone in the clear central region large thiosomes were frequent and the majority of these were distinctly thick, structure-possessing bodies. However, the large and smaller thiosomes with no apparent patterns were most common. Frequently, all sizes were intermingled. In shape, the majority of thiosomes were spherical and not infrequently two of these were so closely united that they were termed diplococoid-thiosomes. Elliptical forms were not uncommon and when of sufficient size, their internal structures were of interest. Thiosomes at times were associated with every colony on an agar plate; sometimes, however, they appeared only with the S or Sr colonies, certainly infrequently with the moderately R type; if they were few in number they occurred only with the growth arising from the initial streak of an inoculating needle. In serial transfers, with little or no indication, they frequently disappeared temporarily,—to return suddenly in small or great numbers. The isolation or crowding of colonies on a plate, as well as their inherent vigor, often influenced the picture. Whenever colonies exhibited the possibility of bacteriophage activity, thiosomes did not appear in the region of the "bitten" areas. Upon making a Gram stain of a smear from an area containing a thiosome, small, highly refractive granules were seen along with the Gram-negative

bacilli; impression preparations were made of thiosomes and one is shown in figure 2; if the cover slip was tapped lightly, when making an impression preparation, the thiosome ruptured and the embedded granules escaped from their confinement within a capsule-like structure (fig. 3).

Conditions for the occurrence of thiosomes were essentially those which were conducive to dissociation. Media used contained several concentrations of NaCl and CaCl_2 and mammalian Ringer's; media to which no salts were added were also used. All of these will be described for each particular series or problem. Emphasis must be placed upon the two kinds of veal infusion media: fresh veal and a dehydrated Bacto-veal infusion. Upon chemical analysis the latter contained 1.5 mgm. of calcium per liter, while the former showed only a trace.¹ Qualitative microchemical analyses were made to detect cations and anions present in both the typical and atypical thiosomes. The formation of thiosomes was influenced by media whose nutritive qualities varied as is shown by a comparison of the atypical thiosomes on a synthetic medium or on fresh veal media with the typical ones when Bacto-veal infusion or fresh veal infusion to which calcium was added was used as a base. The studies of Jordan (1926) and others, relative to interconvertibility of R and S types suggested that rapid transfers be employed and these exhibited much of interest not only in direct relation to thiosomes but also permitted of a correlation between these structures and certain growth characteristics in broth such as surface growth, turbidity, sediment and motility.

The microchemical technique employed for the detection of cations and anions present in the thiosomes will be described later. Figures 6 and 7 illustrate crystals typical of Ca^{++} and SO_3^{--} .

Problems relative to the sources and characteristics of thiosomes and their correlation with cultural features will be treated in the following order:

¹ The author is indebted to Mr. R. A. Green, Assistant Agricultural Chemist, University of Arizona, for the determination of the calcium content of these two infusions.

Series I. Cultural studies of *S. Schottmülleri* filtrates in salt agar and bouillon.

Series II. Thiosomes from *S. Schottmülleri* in serial salt broths.

Series III. Thiosomes from certain other members of the colontyphoid group.

Series IV. Thiosomes from *E. coli* isolated from pathological material.

Series V. The influence of a synthetic medium upon thiosomes.²

MICROCHEMICAL ANALYSES

In an effort to identify cations and anions that might be present in thiosomes it was necessary to resort to "chemical microscopy" as described by Chamot and Mason (1931). Microscopic qualitative chemical analysis demands special technique and within certain limitations permits of results that otherwise could not be obtained. Many of the "micro" tests were not applicable to the present problem due to factors that will be mentioned in connection with individual analyses. The minimum of material available for examination must be emphasized together with the impossibility, with rare exceptions, of its separation from substances of great complexity in the colony and the medium. When tests demand careful control of such factors as temperature, acidity or alkalinity, and concentrations, they become practically impossible under the present conditions. The following factors may give some appreciation of the intricacy of the problem: (1) the complexity of the mixtures of substances in different media; (2) the factors introduced by the metabolic activities of the bacteria themselves; (3) the fewer specific tests for anions than for cations; (4) the possibility that the cation in the unknown might react with an anion in the reagent; (5) the nature of the cation might influence the behavior of an anion toward reagents.

Had it been possible to place thiosomes upon a microscope slide and use the ordinary "micro" tests of decantation, filtration, sublimation, distillations, etc., many difficulties would have been eliminated. Since adequate separation of thiosomes was impossible, the tests were carried out using thiosomes associated with colonies of varying ages on ordinary moist agar plates and also after the agar had been dried at room temperature nearly to the point of cracking. The latter plates gave more clear cut results. The plates were oriented on the stage of a

² I wish to acknowledge my indebtedness to Dr. E. O. Jordan for his assistance and stimulating interest during the progress of this work.

colony microscope and the various reagents added by means of Pasteur pipettes. Magnifications of 40, 100 and 440 diameters were ordinarily used. Identification of cations was carried parallel to the identification of anions. In general, the Bunsen-Treadwell classification of the acids was followed. "This classification is based upon the behavior of the commoner anions toward silver nitrate and toward barium chloride in neutral and in nitric acid solutions" (Chamot and Mason, 1931). Although this particular problem presented certain difficulties, peculiar perhaps to the usual application of microchemical analysis and was consequently subject at times to some limitations, nevertheless the value of such technique should be emphasized. Used singly, tests are often inconclusive but together they permit of the identification of ions with a remarkable certainty and dependability.³

Detection of anions. Tests which indicated the absence of borates, nitrates, nitrites, mono-, di- or tri-alkali phosphates together with hypophosphites and phosphites are not included in this paper because of the limitation of space. There were probably no carbonates of Na and of the K group or most of the other elements present, but due to technical difficulties and the extremely minute amounts of gas evolved when acids were applied, even though no crystals or aggregates were produced, the presence of carbonates could not be completely ruled out. One of the tests suggested orthophosphates but this could not be confirmed with other reagents. According to Chamot and Mason (1931), "salts of SO_4^{--} are seldom contaminated with salts of the other ions of the group, but the salts of the remaining ions ordinarily contain more or less impurities, such as sulphates or salts of the other more closely related ions, the nature of which depends upon the method used for the formation of the ion in question. These foreign ions, which are almost invariably present, introduce a complication in the analysis of very small amounts of material and may trouble the analyst." SO_4^{--} was occasionally indicated in some thiosomes and SO_3^{--} was regularly revealed by the group reactions. BaSO_3 crystals were readily soluble in HCl and typically anisotropic. $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ produced in plates, which did or did not contain thiosomes, tiny disks and spherulites which tended to develop into aggregates. These formed slowly and attained the approximate size of thiosomes. At first, these spheres developed in the medium around the colony margin but after thirty to sixty minutes

³ The author gratefully acknowledges the assistance of Dr. M. N. Short, Professor of Optical Mineralogy, University of Arizona, who verified the identity of the crystals.

they occurred distributed through out the colony area and were almost as large as but less numerous than the thiosomes. These artificial thiosomes or CaSO_3 crystals appeared identical with all previously described thiosomes and where Ca^{++} and SO_3^{--} concentrations were high artificial soot-like thiosomes simulated those produced by *S. Schottmülleri* on CaCl_2 agar plates. Thus, the formation of artificial thiosomes upon addition of calcium clearly indicated the presence of an excess of SO_3^{--} due to bacterial activity. Therefore, calcium present only to the extent of 1.5 mgm. per liter, in Bacto-medium, was insufficient to unite with all the SO_3 ions. The above results were obtained with the Bacto-veal infusion medium without the addition of any salt and also with the NaCl plates. When the above was repeated using the uninoculated control plates no artificial thiosomes occurred. When $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ was added to colonies on M/10 NaCl agar which had fresh veal infusion as a base and hence possessed no thiosomes, artificial thiosomes developed which were identical to those on the Bacto-agar plates (fig. 6). These occurred primarily around the margin of the colony but there were also a few scattered spheres in the central areas of colonies. As a rule, one to two hours was required for appearance. It was of interest to note that in some plates sulphite crystals formed a compact zone around the margin of the colony provided the latter was well isolated; if the colony margin was near another colony, then the artificial thiosomes were scattered and irregular in the peripheral region. This response to crowding corresponded to the way in which the "Schleimwallbildung" phenomenon was noted. Thus, SO_3 ions were present as a metabolic by-product but with insufficient calcium present in fresh veal, which upon analysis showed only a minute trace of calcium, no precipitate could form. When the above was repeated using M/10 NaCl agar which had fresh veal infusion as a base and to which phosphate had been added, using a pH of both 7.2 and 7.8, no artificial thiosomes formed. Does this mean that the presence of phosphate inhibits sulphite crystal formation? Since dibasic potassium phosphate is often added as a buffer and since the commonly used fresh veal has a comparatively high phosphate content, these two factors may account for the failure to date to find these sulphite crystals reported in the literature. By the $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ test, sulphites may be distinguished from thiosulphates since the calcium salt of $\text{S}_2\text{O}_3^{--}$ is too soluble to separate. Thus, this serves as another means of confirming the presence of CaSO_3 . Thiosulphates and persulphates were not detected.

Detection of cations. The chemical tests which established the identity of the predominant anion, namely, sulphite, also suggested calcium as the cation. Tests for many of the other elements likely to be present either proved definitely negative or else the difficulties already pointed out entered to such an extent as to exclude, at least until technique can be further developed, the presence in sufficient quantity of any other element which might play a definite rôle in the formation of thiosomes. Although there is a close relationship between the alkaline earths generally, there are differences in the appearances and solubilities of their salts which permit of differentiation.

Promptly upon addition of sulphuric acid effervescence within the thiosomes occurred. Different concentrations of H_2SO_4 were used and within rather wide limits the same crystals were obtained. After the bubbles had risen to the surface of the test drop, the thiosome appeared to have lost something of its opacity and often highly resembled a translucent or atypical thiosome (fig. 5). The latter was especially true when a concentration of $\text{M}/5 \text{H}_2\text{SO}_4$ was used. After about ten to fifteen minutes the thiosome appeared somewhat fuzzy and crystals typical of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ rapidly formed (fig. 7). Long monoclinic prisms formed with ends which became irregularly and obliquely truncated. Sometimes these prisms gave rise to plates or scales which is a characteristic of Ca^{++} . Photographic records of these changes were carefully made and controls were run with various uninoculated media. Since this test gave such typical crystals, salts of any trivalent metals were excluded, for had these been present they would have interfered with this sulphuric acid test. Although strontium, barium and lead yield crystalline precipitates these could be ruled out with certainty, because of lack of rapid disintegration into a granular deposit, absence of an amorphous precipitate and general characteristics as to appearance and solubilities respectively. The presence of calcium was confirmed by means of the oxalic acid test.

Attempts were made to identify calcium by means of the following reagents: (1) sodium tartrate strongly acidified with tartaric acid, (2) potassium ferrocyanide, and (3) arsenic acid. None of these were satisfactory. This is not surprising since these tests require a moderately high concentration of Ca, which is higher, evidently, than was present in thiosomes; also, since the acidity or alkalinity is of great importance this constituted a factor most difficult of control. Only with the CaCl_2 uninoculated control plates were crystals typical of calcium obtained.

SERIES I. THIOSOMES FROM *S. SCHOTTMÜLLERI*, R TYPE, FILTRATES

In certain bacteriophage studies, carried on with a single-cell culture of *S. Schottmülleri*, 210R₁,⁴ Bacto-veal infusion bouillon and agar,⁵ pH 7.4 and having the following salt concentrations were used: M/1 NaCl, M/2 NaCl, M/10 NaCl, mammalian Ringer's, M/5 CaCl₂, M/10 CaCl₂ and H₂O. The "H₂O" broth or agar simply denotes the total absence of added salts. The 210R₁ culture had been well adapted to these salt concentrations since during the previous eight months it had been used in studies relative to certain dissociative phenomena. In an attempt to ascertain why a relatively high percentage of Berkefeld filtrates in sealed glass ampoules became more or less cloudy or opalescent, the ampoules were well shaken, opened and 5 drops of each transferred to the respective salt broths. From the twenty- to twenty-four-hour-old salt broth cultures, incubated at 37°C., daily motility tests were made, smears for Gram stains prepared and salt agar plates streaked.

Two examples of the characteristics of daily transfers in two salt broths, M/10 NaCl and M/10 CaCl₂, may be briefly summarized as follows: In both media, changes in the surface growth occurred about the 6th transfer and again upon the fourteenth or fifteenth transfers; turbidity reached a maximum on the fourth day and maintained this maximum throughout the remainder of the experiment; the sediment was usually granular and slightly viscid until the seventh to twelfth transfer, inclusive, when it

⁴ Dr. E. O. Jordan's collection.

⁵ Bacto-veal infusion medium, dehydrated, contains veal, infusion from 500 parts; Bacto-peptone, 10 parts; Bacto-agar, 1 part; and the infusion is made in the presence of 1 per cent gelatin. Hitchens (1921) suggested certain advantages of approximately the above medium which was modified by The Digestive Ferments Company, Detroit, Mich. Since in the present investigation it was deemed undesirable to have as much agar present, the dehydrated medium was put into solution by autoclaving, 15 pounds for fifteen minutes, the flasks were placed in the refrigerator over night in order to solidify the agar and the supernatant was carefully filtered through a combined paper and cotton filter. The infusion was made double strength, an equal volume of double strength salt solution was added, titrated, tubed, sterilized (autoclaved, 15 pounds for 20 minutes), and the reaction checked. Agar media contained 2 per cent agar. All media were stored in the refrigerator and care taken to avoid evaporation.

lost its viscosity. The latter returned to a slight degree on the thirteenth, fourteenth and fifteenth transfers; about this time the sediment was also rather flocculent. At about the sixth and again at about the twelfth or thirteenth transfers there was a decrease in the number of motile cells in the M/10 NaCl medium which may also be correlated with findings recorded under series II. The M/10 CaCl₂ showed in both series considerable fluctuations in motility.

In M/10 NaCl, upon first transfer, large, reddish-purple structureless, refractile, coccoid bodies predominated. These became elongated, and large, rather granular, Gram-negative rods subsequently appeared. Pleomorphic forms decreased in numbers in the course of the series of broth transfers and eventually gave rise to text-book-like pictures of *S. Schottmülleri*. As had previously been noted, thiosomes sometimes disappeared for one or more consecutive transfers, suddenly to appear again, and it is of interest to note here that thiosomes were seen in the Gram-stained smear from the broth transfer which preceded the finding of thiosomes on agar plates. It should also be mentioned here that familiarity with these pleomorphic cells permitted the recognition of similar forms, which will be subsequently reported, in connection with the finding of thiosomes associated with an atypical colon bacillus isolated from a case of cervicitis. In M/10 CaCl₂, upon first transfer, large Gram-positive coccoid bodies, which varied in size and shape, predominated. These bodies subsequently were less deeply stained and with them were seen some large Gram-negative rods together with thiosomes which showed the capsule with its internal refractive granules (fig. 2). Cells in these broth transfers were subject to great variations in staining reactions and in morphology, but finally all became definitely Gram-negative; some continued to be somewhat swollen and irregular but most of them were typical of *S. Schottmülleri*.

Thiosomes appeared on the plates streaked from the first 18 transfers in M/10 NaCl broth upon the following days: first, second (not until forty-eight hours incubation), seventh, eighth, ninth (fig. 8), twelfth, seventeenth and eighteenth. This illustrates the irregularity of their appearance especially in the pres-

ence of NaCl, which, together with the changes in growth characteristics in broth, is simply further evidence that bacterial protoplasm is not stable, and that its responses to varying conditions must be studied to a greater extent in their entirety before the probable phases in growth become better understood. Serial transfers constitute one means of accelerating changes that, in the recent past, have gone unrecognized largely due to their suppression because of methods not favorable to dissociation. The thiosomes in M/10 NaCl were never very numerous but definitely well developed. From M/10 CaCl_2 broth, thiosomes were present in every transfer. They were always well developed and abundant in numbers except in the fourth transfer when they failed to appear until forty-eight hours of incubation and were then relatively few in number. The thiosome-colony relationship was very marked, with huge thiosomes superimposed upon the colony in comparison with the small thiosomes in the medium. No elongated thiosomes were seen but the spherical, diplococcoid type was most abundant in the medium surrounding the colonies. From none of the filtrates did the previously mentioned soot-like thiosomes arise.

Attention is called to the thiosomes with the scalloped margins (fig. 8), which have lost their blackness and are less opaque. Thus far, thiosomes superimposed upon colonies only have presented such a picture, but if such a type could be found in the medium surrounding a colony, an impression preparation might reveal more of the actual structure than is seen in preparations more or less confused by ordinary colony cells. When the calcium sulphite is dissolved by suitable reagents, a colony-like structure remains, devoid of any blackness, and with the scalloped margins intact. Whether the bacterial cells that compose this type of margin develop from the colony itself in response to the presence of this crystalline mass, or whether they are the type of cell presumably capable of precipitating CaSO_3 when a sufficient number of such ions are available, remains unanswered. In other words, perhaps the thiosomes are in reality daughter-like colonies.

SERIES II. THIOSOMES FROM *S. SCHOTTMÜLLERI*, R TYPE, IN
SERIAL SALT BROTHS

In the course of certain bacteriophage studies, Bacto-veal infusion agar plates (M/10 NaCl, pH 7.4 to 7.6) were streaked from the daily transfer of 210R₁ in salt broths for the purpose of making observations upon the relative percentages of S, Sr, sR or R colonies which might develop from day to day. These broths contained the same salt concentrations used in series I. The first four daily transfers gave rise to colonies from each broth that were S or Sr in type and of no special interest. No plates were streaked from the fifth, sixth or seventh transfers but upon examination of twenty-hour-old plates from the eighth transfer, extremely numerous thiosomes were seen on all seven plates. Thiosomes appeared upon all plates from the ninth and tenth transfers and two of the latter are illustrated (figs. 9 and 10). Control plates on the media were made and carefully observed concurrently with all transfers but no thiosomes ever appeared upon these sterile plates.

In addition to general characteristics of thiosomes previously given, it was noted that size decreased with either an increase in NaCl concentration or with crowding of the colonies. In shape, the spherical or nearly spherical form predominated and often appeared as a diplococcoid structure (fig. 9). The elliptical thiosomes were not uncommon and were probably most clearly observed from Ringer's broth. It was unusual to find as well developed thiosomes in the agar as are pictured in figure 9. If thiosomes were extremely few in number, they were found where streaking of the plate was begun, i.e., following the line of the first needle tract and here, only, on the entire plate. Occurrence, especially from CaCl₂ bouillon, of very large thiosomes around the periphery of the colony resulted in a perforation of the submarginal area, or an indentation of the edge (fig. 10).

After the appearance of thiosomes upon the eighth transfer, they persisted through the twelfth transfer, but upon the thirteenth transfer they disappeared from certain plates. Observations made upon the thirteenth to eighteenth transfers inclusive are given in (table 1).

TABLE 1

Presence or absence of thiosomes from S. Schottmülleri, thirteenth to eighteenth daily salt broth transfers

TRANSFER	M/1 NaCl	M/2 NaCl	M/10 NaCl	RINGER'S	M/5 CaCl ₂	M/10 CaCl ₂	H ₂ O
13th	—	— ¹	+ ²	— ³	+ ⁴	+ ⁵	+ ⁶
14th	+	+	+	++	+++ ⁷	+++ ⁸	+++ ⁹
15th	— ¹⁰	— ¹¹	++ ¹²	+ ¹³	+++ ¹⁴	+++ ¹⁵	+ ¹⁶
16th	+ ¹⁷	+ ¹⁸	+ ¹⁹	+ ¹⁹	+ ²⁰	— ²¹	+ ²²
17th	— ²³	— ²³	++ ²⁴	++ ²³	+++ ²⁵	+++ ²⁶	++ ²⁷
18th	+ ²⁸	+ ²⁸	++ ²⁹	++ ²⁹	+++ ³⁰	+++ ³¹	++ ³¹

¹ Myriads of shadow-like colonies; small, round, highly transparent, entire margins but rough surfaces.

² Thiosomes only upon initial streaks on plate.

³ S and few Sr colonies.

⁴ S and Sr colonies predominated; few Rs and R.

⁵ Fewer thiosomes than from M/5 CaCl₂; also fewer R type.

⁶ Many shadow-like colonies but not as small or as transparent as from M/2 NaCl; mostly S and Sr. Thiosomes only on initial streaks.

⁷ Some shadow-like colonies like M/2NaCl, thirteenth transfer.

⁸ Thiosomes covered plate; largest perforating thiosomes on initial streaks.

⁹ Few perforating thiosomes.

¹⁰ S and Sr colonies.

¹¹ Sr colonies.

¹² S and Sr colonies. Thiosomes associated with about half of colonies.

¹³ S and few Sr colonies. Thiosomes only on initial streaks.

¹⁴ S, Sr, and R, with tendency toward latter.

¹⁵ Few S; sR and R predominate. Fewer thiosomes than from M/5 CaCl₂. Perforating thiosomes along initial streaks.

¹⁶ S, few sR. Large thiosomes along initial streaks.

¹⁷ S, Sr, few R. Thiosomes along initial streak only.

¹⁸ S, Sr, few Rs. Thiosomes along initial streaks only.

¹⁹ S, few Sr, and many small colonies.

²⁰ S and Sr colonies.

²¹ S, Sr and few R colonies.

²² Sr and sR predominated; some thiosomes in medium adjacent to Rs colonies.

²³ S and Sr colonies.

²⁴ Very thin thiosomes often in clumps. S and Sr colonies.

²⁵ S, Sr few R. Tiny thiosomes in sheet-like growth.

²⁶ R and Sr predominated; few S thiosomes as note 25.

²⁷ R and sR predominated with very few S.

²⁸ S, few Sr. Atypical thiosomes.

²⁹ Like "28" only more numerous atypical thiosomes.

³⁰ Few opaque thiosomes but numerous atypical ones.

³¹ No opaque thiosomes but numerous atypical ones.

Throughout series II, broth characteristics were noted as to surface growth, turbidity, sediment, Gram stains and motility. Since the data accumulated from observations of 21 daily transfers in seven salt broths were so extensive they can only be taken up briefly in the discussion of this paper. Certain of these observations were of interest since they may be correlated more or less closely with each other and with other phases of dissociative phenomena.

In this series as well as others, zones or strata appeared occasionally in old broth cultures. The septa were of a membranous nature, not at all easily disturbed upon agitation, and were not present in M/1 NaCl or in either of the CaCl₂ concentrations. Even though flasks containing the dissolved Bacto-veal infusion medium were placed in the refrigerator for twelve hours or longer, and the extremely low content of solidified agar subsequently removed by filtration, nevertheless there doubtless remained a small amount of agar, so small that it perhaps formed a gel (Hitchens, 1921) which upon standing contracted and settled to the bottom of the tubes. Thus, the layering effect might have been due to growth of bacteria on the surface of the gel alone, or in some instances perhaps a slight pellicle formed and sank to this layer. However, a layering effect was noted in occasional transfers of certain series which, in connection with other work, were made in ordinary broth prepared from fresh veal. Although little has been done thus far, there is an indication that from each zone different types of colonies developed on agar plates when streaked from the upper and lower layers respectively.

SERIES III. THIOSOMES FROM CERTAIN OTHER MEMBERS OF THE COLON TYPHOID GROUP

An attempt was made to find thiosomes in association with certain other members of the colon-typhoid group. The following stock cultures were chosen: *E. coli* "D," 9 *E. typhi*, 131 *S. paratyphi*, 210 *S. Schottmülleri*, and 329 *S. paratyphi*, type C. All were of S type. Fifteen daily transfers were made in two salt media: M/10 NaCl and M/5 CaCl₂ Bacto-veal infusion bouillon and M/10 NaCl and M/5 CaCl₂ Bacto-veal infusion agar (2

TABLE 2

Presence or absence of thiosomes from five members of the colon-typhoid group during fifteen daily transfers

TRANSFER	M/10 NaCl					M/5 CaCl ₂				
	D	9	131	210	329	D	9	131	210	329
<i>1st</i>						++			++	++
24 hours	+++ ¹	—	—	—	—	++	—	—	++	++ ²
36 hours	+++	—	—	—	+ ³	++	++ ⁴	+ ⁴	++	++
						++ ⁴			++ ⁴	++ ⁴
<i>2nd</i>						++			++	++
24 hours	+++	—	—	—	+	++	+ ⁵	— ⁶	++	++
48 hours	+++ ⁷	—	—	—	+ ⁸	++	+	+	++	++
						++			++	++ ⁹
<i>3rd</i>						++			++	++
20 hours	+++	—	—	—	—	++	— ¹⁰	— ¹¹	++	++
48 hours	+++	—	—	—	—	++	+++ ¹²	—	++	++
						++			++	++
<i>4th</i>						++			++	++
20 hours	+++	—	—	—	—	++	+++	+ ¹³	++	++
48 hours	+++	—	—	—	—	++	++	++	++	++
						++	++		++	++
<i>5th</i>						++	++		++	++
24 hours	+++	—	—	—	—	++	++	—	++	++
48 hours	+++	—	—	—	+ ¹⁴	++	++	—	++	++
						++	++		++	++
<i>6th</i>						++	++		++	++
20 hours	++ ¹⁵	—	—	—	—	++ ¹⁶	++	— ¹⁷	++	++
48 hours	++ ¹⁸	—	—	—	—	++	++		++	++
						++ ¹⁹	++ ²⁰	+ ²¹	++	++ ²²
<i>7th</i>							++			++
24 hours	— ²³	—	—	—	—	+++	++	—	+++	++
48 hours	—	—	—	—	—	++	++	+ ²⁵	+++	++
						+ ²⁴	++			++
<i>8th</i>							++		++	++
20 hours	+ ²⁶	—	—	—	—	+++	++	—	++	++
48 hours	+ ²⁷	—	—	—	—	++	++	+	++	++
						+ ²⁸	++		++	++

TABLE 2—*Continued*

TRANSFER	M/10 NaCl					M/5 CaCl ₂				
	D	9	131	210	329	D	9	131	210	329
<i>9th</i>										
24 hours	+ ²⁹	—	—	—	—	+++	+++	— ³⁰	+++	+++
48 hours	+	—	—	—	—	++	++	—	++	++
						++	++		++	++
<i>10th</i>						++			++	++
24 hours	—	—	—	—	—	++	+++	—	++	++
48 hours	—	—	—	—	—	++	+++	—	++	++
						++			++	++
<i>11th</i>						++			++	++
24 hours	—	—	—	—	—	++ ³¹	+++	—	++	++
48 hours	+ ³⁹	—	—	—	+	+++	++	—	++	++
							++ ³²		++	++
<i>12th</i>						++			++	++
24 hours	++ ³³	—	—	—	—	++	+++	—	++	++
48 hours	+++	—	—	—	—	++	++	—	++	++
						++	++		++	++
<i>13th</i>						++	++		++	++
24 hours	+	— ³⁴	— ³⁴	— ³⁵	— ³⁵	++	++	—	++	++
48 hours	+ ³⁶	—	—	+ ³⁷	+	++	++	—	++	++
						++	++		++	++
<i>14th</i>						++			++	++
24 hours	+ ³⁸	— ³⁹	—	—	—	++	+++	—	++	++
48 hours	+	+	—	+	—	++	+++	—	++	++
						++			++	++
<i>15th</i>						++			++	++
20 hours	+ ³⁹	— ³⁹	— ³⁹	— ⁴⁰	— ⁴⁰	++	+++	—	++	++
48 hours	+	+	—	++	++	++	++	—	++	++
						++	++ ⁴¹		++	++

¹ S and few R colonies; latter devoid of thiosomes.² Finest thiosomes yet observed, i.e., the colonies appeared to be buried in soot.³ Two colonies only possessed thiosomes.⁴ Innumerable, fine opaque thiosomes, soot-like.⁵ Numerous soot-like thiosomes on one small area only.⁶ Colonies very small and relatively few in number.⁷ All colonies smooth from all five cultures on NaCl agar.⁸ Thiosomes on only six colonies.

TABLE 2—*Continued*

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- ⁹ Margins of crowded colonies obscured by soot-like thiosomes.
- ¹⁰ Colonies much smaller but relatively numerous.
- ¹¹ Colonies extremely small and few in number in subsequent transfers.
- ¹² Some colonies with large thiosomes, some buried in soot, some which combine previous two types and some devoid of any thiosomes.
- ¹³ Two areas only with dense fine thiosomes.
- ¹⁴ Thiosomes large, elongated and as diplococcoid bodies.
- ¹⁵ Sudden increase in R colonies (one-third of total, fig. 11). Truly R type devoid of thiosomes but a few Rs colonies possessed them.
- ¹⁶ Increase in size and structural changes of thiosomes associated with *E. coli* when seventy-two hours old (fig. 12), and the identical area after twelve days at 37°C. (fig. 13). Generally, there was little or no increase in size on NaCl or H₂O plates after thirty-six to forty-eight hours.
- ¹⁷ Marked differences in size of colonies. In forty-eight hours, thiosomes on large Rs or Sr colonies only (fig. 15, ×40).
- ¹⁸⁻²³ Figures 11 to 16, inclusive, seventy-two hours, ×40.
- ²⁰ Figure 14, *E. typhi*, seventy-two hours. Increase in number of soot-like thiosomes but some colonies strikingly free from them.
- ²¹ Figure 15. *S. paratyphi*, seventy-two hours, ×40. Thiosomes grouped in only few areas on plate. Note translucent and semi-transparent thiosomes among typical opaque ones. Large and small colonies usual at this period and thiosomes only in conjunction with former which were also somewhat granular.
- ²³ Seventy-five per cent colonies rough.
- ²⁴ Figures 17 and 18, ×40. Two typical areas on this plate. Upon this transfer, decrease in relative numbers of thiosomes on CaCl₂ medium but disappearance in NaCl medium. Attention is directed to effect of crowding on thiosomes (fig. 18).
- ²⁵ R and S colonies present; R or Rs colonies only possessed thiosomes. This the only exception found where roughness was not associated with a definite decrease in thiosomes.
- ²⁶ Figure 19.
- ²⁷ Six colonies only with thiosomes.
- ²⁸ After ten days, characteristic daughter colonies; these not uncommon in other old cultures.
- ²⁹ Majority of colonies very rough.
- ³⁰ All colonies uniformly small and smooth.
- ³¹ Figure 20, seven days old. Note atypical thiosomes.
- ³² Similar to figure 20.
- ³³ Ninety per cent R colonies; thiosomes on all types but predominate on Sr and S.
- ³⁴ Few "bitten" areas in colonies. All S or Sr type.
- ³⁵ Many "bitten" areas in colonies. All S or Sr type.
- ³⁶ As twenty-four-hour colonies Sr predominated but when forty-eight hours old, S colonies developed as outgrowths along line of inoculation in certain areas and these possessed thiosomes. In certain Rs colonies, atypical thiosomes (figs. 4 and 5; fig. 20, in submarginal zone).

TABLE 2—*Concluded*

³⁷ Figures 21 and 22. Thiosomes nearer the margin appeared larger and more markedly elliptical. Note internal structure. Purely S colonies were devoid of thiosomes and showed evidence of bacteriophage activity (fig. 22). With exception of *E. coli* all cultures, 9, 131, 210 and 329 showed probable evidence of bacteriophage activity upon this thirteenth transfer.

³⁸ All colonies R or Rs except two S, and latter have "bitten" areas; these "bitten" areas suggestive of bacteriophage activity were devoid of thiosomes. Many R-S colony combinations resembled the "Bombenformen" of Braun and Weil (1928).

³⁹ All colonies smooth and few have "bitten" edges.

⁴⁰ All S or Sr colonies; few "bitten."

⁴¹ Figure 23. S type colony margins only appear when farthest away from other colonies which region contained a few large thiosomes. This phenomenon paralleled characteristic appearance or non-appearance of "Schleimwallbildung," i.e., latter not observed in closely adjacent colonies. Note also that large type of thiosome often inhibited in its vicinity the soot-like thiosomes.

per cent), the addition of calcium serving as a control. Media were prepared as for series II but with a pH of 7.8. No record of daily broth characteristics was kept. From each twenty- to twenty-four-hour-old daily broth culture, salt agar plates were streaked and the presence or absence of thiosomes tabulated (table 2) together with notes and photographs (figs. 11 to 23) of certain findings. Observations were taken regularly at both twenty-four and forty-eight hour intervals during incubation at 37°C. Occasionally records were made of certain changes in colonies that occurred at subsequent intervals. The first transfer plates were accidentally left at room temperature, about 24°C., over night after the first twenty-four hours at 37°C. Direct prints (Buchholz and Lewis (1930)) were made of the first transfer plates and areas typical of each were ringed and photographed at a magnification of 75. Photographic records were resorted to when marked changes occurred. A magnification of 40 was used for all except the first transfer.

The presence of thiosomes in conjunction with five members of the colon-typhoid group, throughout, or at certain intervals during, 15 daily transfers and their relative number from day to day on M/10 NaCl and M/5 CaCl₂ agar plates has been of much interest. On the control CaCl₂ plates, thiosomes could be noted

upon macroscopic inspection of the thirty-six-hour-old first transfer Petri plates by the cloudy areas adjacent to and including the colonies; microscopically, thiosomes were so numerous that colony margins were more or less obscured. In the footnotes of table 2 details are given with emphasis on the type of thiosome found.

SERIES IV. THIOSOMES FROM PATHOLOGICAL MATERIAL

The finding of thiosomes in association with *E. coli* isolated from a case of cervicitis was of considerable interest and points toward further investigations that should be carried out with pathological material. This patient had been undergoing treatment for a month and was not showing as rapid improvement as was expected; it was at this time that the following work was done. During the fifth week three specimens were sent to the laboratory and the results from each, herein reported, were in agreement.

This case had been reported by a technician in a local commercial laboratory as a streptococcus infection; the clinical picture contraindicated this diagnosis in the judgment of the physician. It was found that the technician made his report solely on a Gram-stained smear from a six- to eight-hour-old brain broth culture (Rosenow's formula, 1928). To check his report, the specimens herein reported were likewise inoculated into brain broth together with certain other media: nutrient broth, glucose broth containing a piece of fresh, sterile, rabbit kidney, nutrient agar, Endo's medium and veal infusion rabbit blood agar plates (both streaked and poured). Several direct smears were also obtained from the patient and stained by Gram's method. After an eight-hour incubation period in brain broth, Gram-stained preparations revealed the presence of pleomorphic cocci; the predominating type was a huge Gram-negative diplococcus which occurred frequently in chains made up of distinct diplococci. The direct smear showed this same form together with a few typical staphylococci, a few large Gram-positive rods, and some Gram-negative coli-like bacilli. From both preparations, direct smear and brain broth, the large diplococcus was less easily decolorized by alcohol than most Gram-negative organisms; this

was checked time and again with known positive and negative cultures until no hesitancy existed in reporting it as Gram-negative. With the exception of very few typical staphylococci, nothing but coli-like rods were found in other media. Transfers to sugars gave *E. coli* characteristics. After eighteen hours at 37°C. in brain broth, very few coccus-like cells were seen and these were never in chains. On Endo plates, the characteristic metallic luster of *E. coli* developed only upon forty-eight hours incubation. Transfers from brain-broth cultures to nutrient, blood, and Endo agar plates revealed nothing but colon-like colonies with an occasional typical staphylococcus and also a few colonies made up of large Gram-positive rods. When sugars were inoculated with three slant cultures of coli-like bacilli, obtained from the atypical Endo plate colonies, one of these slants was placed upon the stage of a microscope and observed under low power. Typical thiosomes were numerous and were also present in the other two Bacto-nutrient agar slants. While this is a report upon three specimens from only one case, this came at the time when thiosomes were the objects of much interest, and offers another distinct source of calcium sulphite crystals precipitated by a member of the colon-typhoid group.

In connection with pathological material, the fact is recalled that when bacteria are isolated from the centrum of certain calculi the colon and typhoid bacilli are most frequently found (Wells, 1925). The possibility that certain variant bacterial cells may have the ability to precipitate calcium and hence be of significance in the formation of certain concretions in the animal body will be discussed briefly in this paper.

SERIES V. INFLUENCE OF A SYNTHETIC MEDIUM ON THIOSOMES

An attempt was made to determine whether or not thiosomes would develop in conjunction with R and S strains of *S. Schottmülleri* when a synthetic medium was used with the same concentrations of NaCl and CaCl₂ employed with the filtrates in series I. Single-cell cultures used were those above referred to as 210R₁ and 210S₂; the former had been cultivated for some time in Bacto-veal infusion salt broths, but the latter was merely a well-

cared for stock culture. The medium used was the "number eight"⁶ reported by Stuart (1924). It will be noted that the only possible source of sulphur ions was in the magnesium sulphate, and calcium was available only in the one type of medium. Seventeen daily transfers from broth to broth and broth to agar plates were made. Observations were made as reported for the previous series except that plates were incubated for seventy-two hours at 37°C.

The R and the S strain grew far less luxuriantly than in veal infusion broths and the colonies on plates were always small. At irregular intervals growth was so scanty that certain of the higher salt concentrations had to be reseeded from a lower concentration in which a more vigorous growth was present. Considerable data accumulated from observations on 210R₁ during seventeen daily transfers in six synthetic salt broths together with six synthetic salt agar plates, and will be summarized as follows: With the R strain, 210R₁, in several synthetic salt broths, slight changes occurred in surface growths upon the ninth to tenth transfers in M/2 NaCl (hazy type of pellicle), M/10 NaCl (for the often found hazy type was substituted a moderate, white pellicle with a granular ring) and M/10 CaCl₂ (instead of irregular variations between an entire absence of pellicle formation or the thin transparent type a slight ring pellicle developed); the ninth transfers also gave rise to a somewhat granular sediment in M/10 NaCl. Changes in colonies occurred approximately at the seventh and fourteenth transfers in M/2 NaCl (S colonies became R; latter were granular on fourteenth and growth disappeared on fifteenth and sixteenth transfers), Ringer's (S colonies became R on eighth transfer and reverted to S upon fifteenth transfer), M/5 CaCl₂ (S colonies gave rise to R on fifth transfer; largest S possessed translucent thiosomes but subsequent R only few and small; growth disappeared tenth to sixteenth transfers when very R colonies appeared which were devoid of thiosomes), and M/10 CaCl₂ (S colonies with translucent or atypical thiosomes present

⁶ Asparagin 0.34 gram; ammonium lactate 1.0 gram; sodium chloride 0.50 gram; magnesium sulphate 0.02 gram; calcium chloride 0.01 gram; potassium phosphate 0.10 gram; glycerol 4.00 grams and agar solution 100.00 cc. For the NaCl broths, CaCl₂ was omitted and vice-versa. pH 7.8.

until fourth transfer when growth suddenly ceased; upon fifteenth transfer R colonies appeared). Atypical thiosomes appeared in both CaCl_2 concentrations only: in $\text{M}/5$ CaCl_2 during the first to the ninth, and in $\text{M}/10$ CaCl_2 during the first to fourteenth transfers.

With the smooth strain, 210S₂, in the salt broths, certain changes occurred in surface growth: about the sixth to seventh transfers in Ringer's (the usual hazy or thin type of pellicle absent), $\text{M}/5$ CaCl_2 and $\text{M}/10$ CaCl_2 (thin transparent pellicle disappeared upon sixth transfer to return twice upon irregular intervals); and again about the fourteenth transfer in $\text{M}/2$ NaCl (only appearance of a slight pellicle), $\text{M}/5$ CaCl_2 and $\text{M}/10$ CaCl_2 (return of thin, transparent pellicle). The ninth transfer showed a granular sediment in $\text{M}/2$ NaCl , $\text{M}/10$ NaCl and Ringer's. Changes occurred in the colonies upon the following approximate transfers: fifth, Ringer's (S colonies became R upon fifth and sixth transfers and then reverted to S), $\text{M}/5$ CaCl_2 (S colonies with irregular atypical thiosomes until fifth transfer when growth ceased; sixth transfer, small R colonies with few atypical thiosomes), and $\text{M}/10$ CaCl_2 (S colonies with atypical thiosomes until fourth transfer, when R colonies devoid of thiosomes appeared; sixth transfer, reversion to S with atypical thiosomes); sixth, $\text{M}/10$ NaCl ; approximate eighth, $\text{M}/10$ NaCl (R to S), Ringer's (R to S upon seventh transfer), and $\text{M}/5$ CaCl_2 (growth disappeared ninth to thirteenth transfers); and fourteenth, $\text{M}/5$ CaCl_2 (small R colonies appeared) and $\text{M}/10$ CaCl_2 (small R colonies appeared). Thiosomes appeared upon the following transfers: first, $\text{M}/2$ NaCl ; first to third and sixth to ninth, $\text{M}/5$ NaCl_2 ; first to third and fifth to twelfth, $\text{M}/10$ CaCl_2 . It must be noted that the thiosomes were not typical, i.e., at no time did the opaque black type appear but only the translucent form (fig. 5 or fig. 20, submarginal zone). Little work has been done with impression preparations of these but they too are atypical.

DISCUSSION

It is evident from the literature which has accumulated that bacterial dissociation involves complex processes which are little understood and difficult of analysis and that bacterial protoplasm

has not the stability accredited to it by conceptions of the recent past, but responds in various ways to the inconstant physico-chemical changes taking place in its environment. During the course of studies on dissociation of certain paratyphoid bacilli, variant cells capable of giving rise to calcium sulphite crystals were found and herein are called thiosomes. These are referred to as thiosomes because indications to date point toward a type of variant cell associated with the sulphite crystals that probably goes to make up a colony-like structure. Their extremely close association with ordinary colonies makes attempts at cultivation most difficult but work is at present under way relative to this phase of the problem.

These variants grew from *S. Schottmülleri* filtrates (series I) in sealed glass ampoules which had become cloudy or opalescent, and also from the following sources: *S. Schottmülleri* in serial salt broth transfers which had not undergone filtration (series II); serial transfers of *E. coli*, *E. typhi*, *S. paratyphi*, *S. Schottmülleri*, *S. paratyphi*, type C (series III); *E. coli* isolated from a case of cervicitis (series IV); and atypical forms associated with *S. Schottmülleri*, single-cell R and S strains, in a synthetic medium (series V). In connection with the above, as well as with certain bacteriophage studies carried on during the past two years, daily transfers in broth have been made in a number of series of several paratyphoid cultures and in each instance certain characteristics appeared and disappeared with sufficient frequency to arouse interest relative to possible correlations of growth phenomena. Gram-stained smears were made daily from each broth and the definite changes in morphology at various intervals will be reported at a later date.

The media used have been fully described and the salts present have doubtless played an important rôle in these studies. Literature which deals with salt effects is extensive and since it has been admirably summarized and analyzed by Falk (1923), Buchanan and Fulmer (1930, vol. II) and others, it demands but few details here. As Fabian and Winslow (1929) point out, all electrolytes, studied by them, stimulate bacterial growth in low and inhibit it in higher concentrations. They found maximum stimulation to

occur with a "Na concentration of 0.10 M and pH of about 7.5;" thus, in the present work, M/10 NaCl, pH 7.4 to 7.8, served as a control as well as did the media to which no salts were added. Their investigation also indicated that a rapid "change from stimulation to inhibition" occurred when Na was increased. In this event, dissociation should be more marked in the M/1 NaCl and M/2 NaCl media; this was found to be true as shown culturally and also by a decrease in the number of motile cells. In a simple synthetic medium, Winslow and Dolloff (1928) showed that cations do not function qualitatively but only quantitatively and effects produced at given concentrations varied decidedly. In the present experiments, M/10 and M/5 CaCl_2 sometimes seemed to inhibit but at other times to accelerate growth to a greater or less extent; certainly, their effect upon different cultures varied, for example, in the scanty growth to which *S. paratyphi* gave rise. Just what the quantitative relationship is between NaCl and CaCl_2 in respect to dissociative changes would have to be ascertained by the use of a greater range of concentrations for each individual culture. Winslow and Haywood (1930) found by means of comparative tests, that stimulation and inhibition concentrations are about the same in distilled water, Dolloff's synthetic medium, or peptone solution. They obtained relative potency constants for eight cations and found that Ca is nine times more potent than Na. Bordet (1930) and Bordet and Renaux (1930) found that calcium constituted an important factor in the production of variants of the anthrax bacillus and they refer to changes induced in certain other bacteria relative to pigment production and morphologic variations when the calcium content of a medium was altered.

Although certain concentrations of NaCl exerted an effect upon the metabolic activities as manifested by variations in precipitation of sulphite crystals, it must be emphasized that other factors, doubtless much more deeply seated, are involved since thiosomes occurred repeatedly in media to which no salt had been added. There is much evidence to show, for example, some effect of S and R colony types on thiosomes or vice-versa; in the great majority of instances some S constituent or constituents seemed

to be necessary for thiosome occurrence (fig. 19) in fact, to date no extremely rough colony has been seen associated with thiosomes. In the synthetic medium, with R constituent or constituents probably present to a greater or less extent, there seemed to be created conditions favorable to the continuation of the formation of thiosomes. Not only did the S strain produce thiosomes intermittently in contrast to their constant presence with the R strain, but the S strain also was associated with thiosomes one transfer less in the M/5 CaCl_2 and two transfers less in the M/10 CaCl_2 than was the case with the R strain. Let it be recalled that the sulphur ions introduced in the form of magnesium sulphate were not found to be transformed into a sulphite so that atypical thiosomes only were formed.

Dissociative changes were probably accelerated by the use of a base for the salt bouillon and agar which was rich in nutritive constituents and retarded by the base in the synthetic medium which was poor in nutritive constituents. Since the synthetic medium permitted only a minimum growth, the colonies were always small and markedly transparent; S and R types were not clear cut and it is worthy of note, that under such apparently adverse conditions, thiosomes developed, atypical though they were. Adaptation of the two cultures to this medium probably would have been of little value, since in connection with other studies, this same medium was used for serial broth-to-broth and broth-to-agar transfers for over three months with little or no improvement in vigor of growth.

Observations indicate that thiosome formation is in some obscure way related to or influenced by bacteriophage. Thus far, sulphite crystals have not been found associated with areas on or near colonies where there is evidence of bacteriophage activity (figs. 17 and 22). Perhaps certain bacteriophage types could be found as suggested from the work of Burnet (1929) and Burnet and McKie (1930). Certainly on agar plates, at least, thiosomes in the great majority of instances are produced by comparatively young colony cells; they are usually associated with colonies on agar plates eighteen to twenty-four hours old, certainly not older than about forty-eight hours, with the exception, at times of the

atypical thiosomes on veal-infusion agar only. The latter appeared likewise with young cells on synthetic media and in the series of transfers reported there was no evidence of phage activity. There are undoubtedly factors which perhaps condition the metabolism of the colony to permit of the development of thiosomes, in fact, even permit at times the development of a definite type of thiosome such as the large elliptical ones.

Since the veal infusion and synthetic media provoked such variations in the response of the organisms to these highly nutritive and slightly nutritive bases, as well as to wide differences in NaCl and CaCl₂ concentrations, it is evident that general correlations relative to growth characteristics must be made with certain reservations. The two media must be dealt with separately. In veal infusion broths, with few exceptions, surface growth of some type was distinct approximately through first five transfers; about this time pellicles changed in character or sometimes disappeared. For the next five or six transfers growth characteristics of the initial three or four transfers generally prevailed. Thus, about the fifth transfer is suggestive of a first critical period and at about the 13th transfer a second such period was often evident.

These fluctuations in veal infusion broth surface growth characteristics can be correlated to a certain extent with motility. With the exceptions of the broths with the greater salt concentrations, a high percentage of motile cells was observed until about the first critical period when there was an abrupt decrease. This was also true about the twelfth to fourteenth transfers and in the interim, although there were variations, the trend was toward an increase in the percentage of motile cells. No motility tests were made in the serial broth transfers with the synthetic salt broths. Motility investigations have been of much interest since a motility-temperature relationship (results as yet unpublished)⁷ was found to exist among some of about 160 stock cultures of paratyphoid bacilli.⁸ The work herein reported as well as the literature that will be presented in a subsequent report relative

⁷ Jordan, E. O., and M. E. Caldwell.

⁸ Dr. E. O. Jordan's collection.

to this temperature-motility relationship, suggests that besides temperature or its influences, lack of motility may be due to an interval in a dissociative recurrent change when a type of cell predominates that either lacks flagella or possesses non-functional flagella.

The appearance or disappearance of thiosomes has been detailed and some sort of correlation between these bodies, colony variations, broth characteristics and the relative number of motile cells is suggested. In series II, the appearance of thiosomes when both surface growth and motility were inhibited by the high salt content of M/1NaCl, indicates an inherent potency or potencies which were not entirely suppressed by the environmental conditions imposed upon the organisms in these media.

From observations thus far made, there seems to be considerable indication that perhaps thiosomes are precursors of or in some way related to variant colonies which have been described by Hauduroy (1927), Vaudremer (1921), Soule (1928), Hadley (1931a) and others. The first evidence of this arose in connection with the filtrate studies, series I. The scalloped translucent margin of thiosomes previously described (fig. 8), may have developed in response to the presence of CaSO_3 crystals but its occurrence might also be interpreted as a growth of variant cells in the colony-like structure that progressed after all available Ca^{++} and SO_3^{--} had united and therefore this newer growth lacked opacity due to the absence of sulphite crystals. What have been called atypical thiosomes were first observed in series II, but were studied in greater detail in series III (fig. 20, submarginal zone) and series V. Frequently associated with these on agar plates were transparent, extremely tiny to small colonies. *S. paratyphi* (series III), sixth daily broth transfer, after seventy-two hours incubation at 37°C . on M/5 CaCl_2 agar (fig. 15) gave rise to thiosomes that were of much interest primarily because of their variations in opacity. Those that either did not possess or had lost their greater degree of opaqueness or blackening showed differences in internal structures varying from extreme roughness to comparative smoothness; and of still greater interest were the innumerable tiny transparent colonies.

In keeping with the work which has shown that dissociation may be induced by salts and by differences in nutritive values of the medium and thereby give rise to sulphite crystals, it was not a surprise to find thiosomes associated with an atypical *E. coli* isolated from a case of cervicitis (series IV). It is not known whether the *E. coli* isolated was the etiological agent, but it is suggested that since this patient had received treatment three times a week for a month and had shown an improvement, the organism might have been affected not only by the condition of the host but also by the medicinal applications to which it had been subjected. Although this is a report of a single case, it aroused much interest not only because the thiosomes were worthy of considerable study, but also since the cells from direct smears and from young brain broth cultures, were similar to those seen upon first examination of sealed cloudy filtrates (series I) and which also developed into "normal" *E. coli* cells. It is planned to continue this work when material can be obtained from similar infections and secured prior to, as well as at intervals during, treatment.

It is suggested that the formation of some of the clinically important concretions in the animal body may be related to the ability of certain variant bacterial cells to precipitate calcium. In a review of the literature it is found that much uncertainty is expressed relative to the rôle which infection may play. Many citations that seem to have a distinct relation to the present work might be made but it is not within the scope of this paper to discuss the complex physico-chemical factors involved. In man, the most common calculi are those found in the biliary and urinary tracts and they are frequently associated with inflammations. While the nidus or centrum is frequently a mass of desquamated cells, fibrin, mucin or some type of foreign body, it has also often been found to be a clump of bacteria (Karsner, 1929), the colon and typhoid bacilli being most frequently incriminated, and bacilli have been found viable in gall stones for surprisingly long periods of time (Wells, 1925).

Concretions usually consist of a mixture of the materials present but are never composed of one substance in a pure form. Interest

here centers in the ever-present calcium salts of the bile pigments which are always found even though cholesterol is present to such an extent as to be referred to as a "pure" cholesterol stone (Wells, 1925). Besides the combination of calcium with the pigments, such as bilirubin and biliverdin, this element has been reported, for example, as a carbonate, phosphate, sulphate, and even oxalate (Wells, 1925). In the blood, proteins hold the calcium salts in solution or suspension in an unstable condition. Many hypotheses exist but an explanation of the causation of stones is still to be found. Lichtwitz (1910) has shown that when the gall bladder is inflamed calcium and magnesium are precipitated along with cholesterol, bilirubin and proteins. Since calcium is derived from the blood and is found to be present to the extent of about 0.1 gram per liter of serum (Wells, 1925), whereas in Difco-veal-infusion medium calcium was precipitated as a sulphite when only 0.0015 gram calcium per liter was found, it seems possible in the light of the present work that variant bacteria which may be present in such infections might find such an environment favorable for the precipitation of calcium with the consequent formation of calculi.

In support of this possibility, certain experimental biliary tract disturbances have been induced which resulted in changes in the calcium content (Wilkie, 1928; Andrews and Hrdina, 1931). Rous, McMaster and Drury (1924, I) induced lithiasis in dogs by intubation for the collection of sterile bile. They avoided stasis and state that infection played no part. Of their 22 experimental dogs, 35 per cent of the 14 dogs with stones were not used because of infections. No data were given as to the bacteria found in the infections. Even though the secretions were sterile in the dogs studied, is it not conceivable that in certain locations as the lower part of the cannula "coated with organic débris and in the midst of . . . calculi," there might have been bacterial variants not readily cultivable, not responsible for a frank inflammation and yet capable of playing some part in the precipitation of calcium salts normally present? Stone formation seemed to be definitely associated with the presence of organic débris and the authors statement that "calculi never developed on the surface

of the material but always within it as if determined by some special condition there" suggests in light of the present investigation some type of bacterial activity. Of interest in this and especially in a second paper (Rous, Drury and McMaster, 1924, II), is the description of the nuclei of stones. Many types of calculi were found to contain minute spherical nuclei which in their photographs, plate 17, figures 9 and 10, appear most similar to thiosomes. Since these nuclei effervesced but slightly when treated with dilute HCl they were thought to contain only a small amount of carbonate; however, the present work would suggest that this reaction may have been due to the liberation of SO_2 . Rous, et al. (1924, II) state that "whole crops of stones were sometimes present side by side. It was as if at some critical period in bile collection bilirubinate had come out of solution providing here and there centers favorable to subsequent layering with another material. . . . The question arises as to the possibility of a correlation between the "crops" and the often sudden appearance and disappearance of thiosomes in the present investigations. When CaCl_2 was injected intravenously into the dogs a "shower" of nuclei sometimes occurred. This might be correlated with the finding of the enormous numbers of soot-like thiosomes when CaCl_2 was purposefully added to media. The authors note that in bile from human gall bladders with concretions, calcium carbonate spheroliths were found and thought to act as nuclei for the formation of secondary stones.

At this time, no attempt can be made to cite the results of other investigations which indicate the possibility that bacterial dissociants may have been involved in the formation of calculi. Under various pathological conditions there are records of the inability to culture bacteria when observed microscopically, of irregularly stained cells, of loss of viability under certain conditions, morphological, colonial and cultural variations together with lack of motility of certain bacilli all of which are suggestive of the above hypothesis (Mackey, 1931; Martin, 1929; Nauss, Lake and Torrey, 1931; Judd, 1927; Wagner, 1922; Branch, 1929; Nagai and Watanabe, 1927; Olivieri, 1929; and Kelly and Dible, 1930).

In the growth and metabolism of bacterial cells in general sulphur is apparently an essential element (Buchanan and Fulmer, 1930, II). Most cells assimilate this in the form of sulphates although other compounds are sometimes utilized. Much of the work reported involves studies of H_2S production by bacteria, as well as some other sulphur compounds, but few references have been found relative to the formation of sulphites. Since normal sulphites slowly add on oxygen, and sulphate enters as an impurity this may have obscured its detection at various times; also, the identification of a sulphite might have been possible had microchemical technique been taken advantage of in bacteriological work to a greater extent than the literature indicates. Cystine is present in almost all bacteriological peptones (Buchanan and Fulmer, 1930, III) and is doubtless the source of various sulphur compounds. Tilley (1923b) stated that "commercial peptones have been shown to contain unoxidized, partly oxidized and oxidized sulphur in varying proportions" and found variations in the amount of hydrogen sulphite produced by bacteria. This paper, as well as a second one (1923a), also suggests the possibility of the presence of variant bacterial cells. Almy and James (1926) in their studies concluded that decomposable sulphur compounds in peptones were promptly utilized and H_2S formed during the stage of rapid multiplication of bacteria. In the present investigation, this early utilization may be correlated with the usual appearance of calcium sulphite crystals in association with relatively young cells. Almy and James (1926) also found variations in the amount of H_2S produced from five brands of peptones commonly used in laboratories; it would be of interest to know how "Bacto-" peptone was valued. Their statement that the rate of H_2S formation was not uniform with *S. aertrycke* or *Proteus vulgaris* suggests that certain dissociative factors may have been active. This variation in rate, should it be found to be applicable to sulphite production, might account for the fluctuations in the number and rather decided differences in appearance that thiosomes exhibited from time to time. In other experiments, when proteolysis was inhibited, more or less, by the addition of glucose, a principle thoroughly investigated

by Kendall and his colleagues (1912), calcium sulphite was not precipitated which indicates the retardation of N metabolism with its consequent prevention of the utilization of sulphur-containing derivatives of protein by the bacteria. McLeod (1928) points out that bacteria capable of forming H_2S probably do so more readily if cystine is added to the medium and yet, there is evidence to the contrary; that some of the coliform bacteria have this ability is recognized by many workers.

By more or less controlling the constituents in media responsible for the formation of $CaSO_3$ crystals, something of an insight into the activities of certain members of the colon-typhoid group may be gained but let it be emphasized that this does not necessarily mean that the same abilities are manifest in the very different environment of an animal body. However, there are several well known conditions that might show some correlation. Patients with cystinuria frequently develop concretions in the kidneys or bladder and sulphates and other neutral sulphur compounds are present in the urine (Wells, 1925). Under such conditions may not the formation of these calculi be incited in part, perhaps, by bacterial activities? Since concretions are often associated with abnormal intestinal putrefaction of protein and since the amounts of ethereal sulphates in urine indicate the extent of this intestinal putrefaction (MacLeod, 1922) it seems possible that certain variant bacterial cells in the urinary bladder might form sulphite crystals; this also might occur in the biliary tract provided ethereal sulphates which are probably formed in the liver (MacLeod, 1922), also find their way into bile. In the gall bladder, therefore, the nuclei already referred to in connection with the work of Rous, et al. (1924) may have had their inception in the form of calcium sulphite crystals as a nidus upon which calcium salts or cholesterol may have been deposited. In the biliary duct system when inflammation leads to a lessened motility with its consequent greater accumulation of organic debris than is normal, then such an environment probably becomes more favorable for bacterial activities. These activities are doubtless little understood and are taking place where perhaps sulphur containing derivatives are not only abnormally high, but furthermore

in an environment where tissues absorb certain sulphur ions at times with great difficulty (Hawk and Bergeim, 1927). The results of this investigation indicate that with certain members of the colon-typhoid group the metabolism of calcium and sulphur is modified by dissociative phenomena.

SUMMARY

This paper deals with what are thought to be variant bacterial cells which have the ability to precipitate calcium sulphite in a suitable environment. Since these variant cells seem to give rise to a daughter-colony-like structure they are termed thiosomes. Two types of the latter are described.

Data are presented relative to the finding of thiosomes from the following sources: sealed filtrates of *S. Schottmülleri* (series I); serial salt broth cultures of *S. Schottmülleri* used in certain bacteriophage studies (series II); serial salt broth cultures of *E. coli*, *E. typhi*, *S. paratyphi*, *S. Schottmülleri* and *S. paratyphi*, type C (series III); *E. coli* isolated from a case of cervicitis (series IV); and atypical thiosomes associated with *S. Schottmülleri* in a synthetic medium (series V).

Evidence that the fundamental structure of a thiosome is colony-like appears as follows: (1) Atypical thiosomes resemble the opaque thiosomes in size, in structure, in position on the colonies and in their irregular occurrence. With polarized light atypical thiosomes do not appear to be of a crystalline nature. (2) Lack of any definite pattern of sulphite crystals when numerous and, when few in number, their formation on the initial streak of the plate only, suggest the presence of scattered variant cells. (3) Intermittent occurrence of thiosomes during serial transfers in the same lots of media to which either no salts or NaCl had been added; also, variations in numbers of thiosomes in the control CaCl₂ media. (4) The association of thiosomes with S and R colony types. (5) The development on the part of some thiosomes of scalloped margins which have either lost their blackness or are less opaque. (6) By application of suitable reagents, CaSO₃ crystals may be dissolved and leave a colony-like structure devoid of any blackness.

Conditions which promote dissociation seem to be conducive to the development of thiosomes, such as age, salts, rapid transfers in broth, composition of medium, and probable activity or activities of bacteriophage. These thiosomes were found, however, in media which contained the usual NaCl concentration, about $m/10$ NaCl, as well as in media to which no salt had been added, which indicates an inherent potency or potencies which, within certain limitations, may not be under the control of environmental conditions.

In serial broth transfers, recurrent changes occurred relative to surface growth, turbidity, sediment and motility. Agar plates streaked from these serial broth cultures indicated that, in the latter, thiosomes were concerned in these changes together with the variations in S, Sr, and Rs and R type colony characteristics.

The possibility that certain variant bacterial cells may have the ability to precipitate calcium and hence be of some significance in the formation of certain concretions in the animal body is discussed.

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PLATES

PLATE 1

FIG. 1. Thiosomes associated with *S. Schottmülleri* colonies, from sealed m/10 NaCl broth filtrate; grown on m/10 NaCl agar; twenty-four hours 37°C. $\times 15$.

FIG. 2. Thiosome, Gram stain, impression preparation. Note granules within capsule-like structure. $\times 1540$.

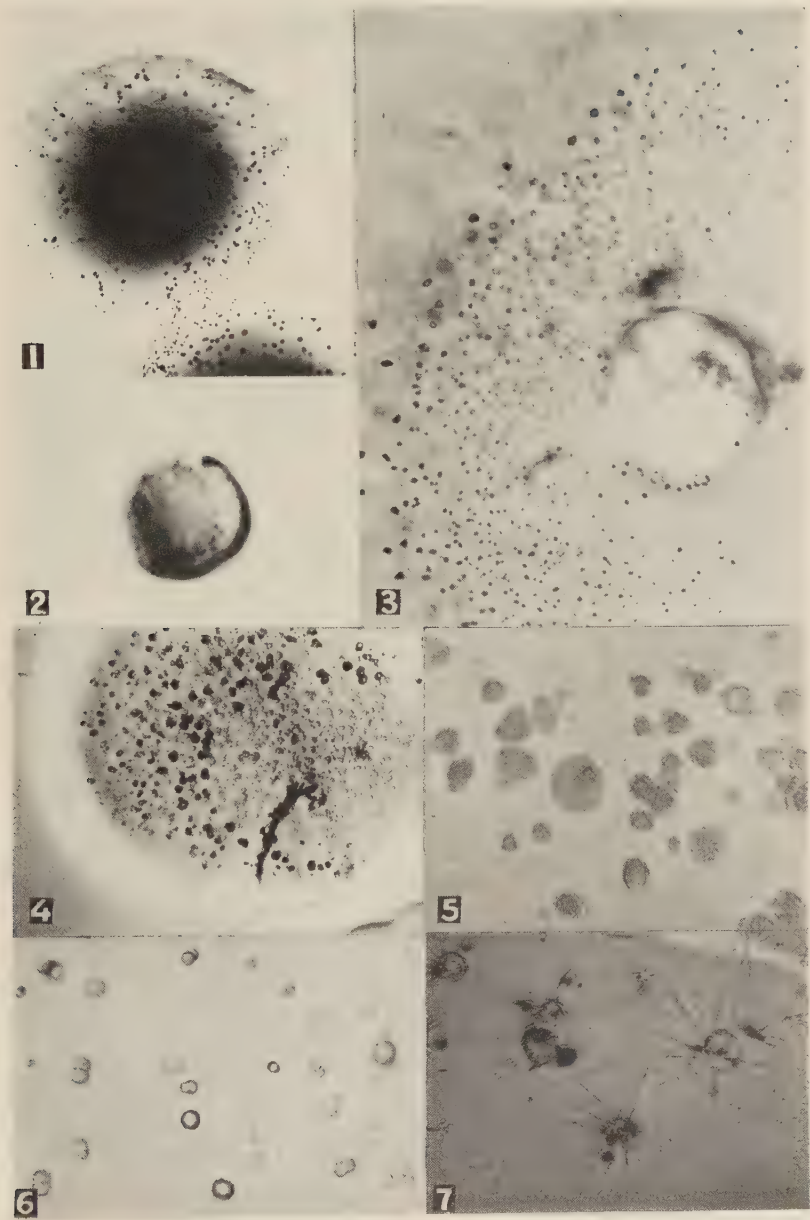
FIG. 3. Ruptured thiosome, Gram stain, impression preparation. Note capsule-like structure from which embedded granules have escaped. $\times 1540$.

FIG. 4. *S. Schottmülleri*, Bacto-H₂O agar; forty-eight hours 37°C. $\times 20$. Typical (opaque) and atypical or translucent thiosomes. Note greater number of atypical thiosomes within segment of colony.

FIG. 5. *S. Schottmülleri*, fresh veal infusion m/10 NaCl agar. $\times 100$. Atypical thiosomes superimposed upon a colony.

FIG. 6. Background of this photograph is surface of *S. Schottmülleri* colony. Fresh veal infusion m/10 NaCl agar. $\times 100$. This colony was devoid of any type of thiosome; upon addition of $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$, CaSO_3 crystals were produced.

FIG. 7. *S. Schottmülleri*, Bacto-H₂O agar, fourth transfer. $\times 100$. Opaque thiosomes treated with H_2SO_4 showed presence of calcium in the formation of these typical CaSO_4 crystals.



(Mary E. Caldwell: Disociation of Paratyphoid Bacilli)

PLATE 2

FIG. 8. *S. Schottmülleri*, from sealed m/10 NaCl broth filtrate. Growth on m/10 NaCl agar after 10 daily transfers in m/10 NaCl broth; twenty-four hours 37°C., plus fourteen days 8°C. $\times 75$. Series I.

FIG. 9. *S. Schottmülleri*. Growth on m/10 NaCl agar after 10 daily transfers in m/10 NaCl broth; eighteen hours 37°C. $\times 75$. Series II.

FIG. 10. *S. Schottmülleri*. Growth on m/10 NaCl agar after 10 daily transfers in m/10 CaCl₂ broth; eighteen hours 37°C. $\times 75$. Series II.

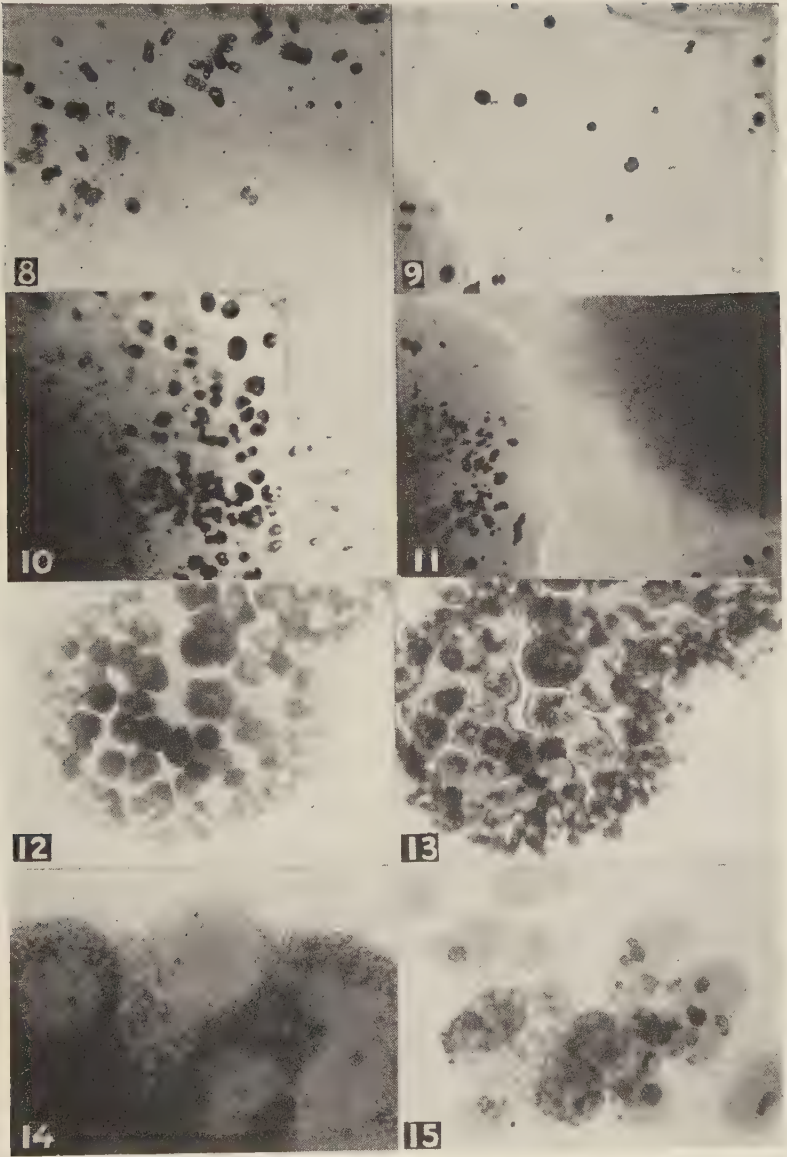
FIG. 11. *E. coli*, m/10 NaCl agar, sixth transfer; seventy-two hours 37°C. $\times 40$.

FIG. 12. *E. coli*, m/5 CaCl₂ agar, sixth transfer; seventy-two hours 37°C. $\times 40$.

FIG. 13. *E. coli*, m/5 CaCl₂ agar, sixth transfer; twelve days 37°C. $\times 40$. Same field as figure 12.

FIG. 14. *E. typhi*, m/5 CaCl₂ agar, sixth transfer; seventy-two hours 37°C. $\times 40$.

FIG. 15. *S. paratyphi*, m/5 CaCl₂ agar, sixth transfer; seventy-two hours 37°C. $\times 40$.



(Mary E. Caldwell: Dissociation of Paratyphoid Bacilli)

PLATE 3

FIG. 16. *S. paratyphi*, type C, m/5 CaCl_2 agar, sixth transfer; seventy-two hours 37°C . $\times 40$.

FIG. 17. *E. coli*, m/5 CaCl_2 agar, seventh transfer; forty-eight hours 37°C . $\times 40$.

FIG. 18. *E. coli*, m/5 CaCl_2 agar, seventh transfer; forty-eight hours 37°C . $\times 40$.

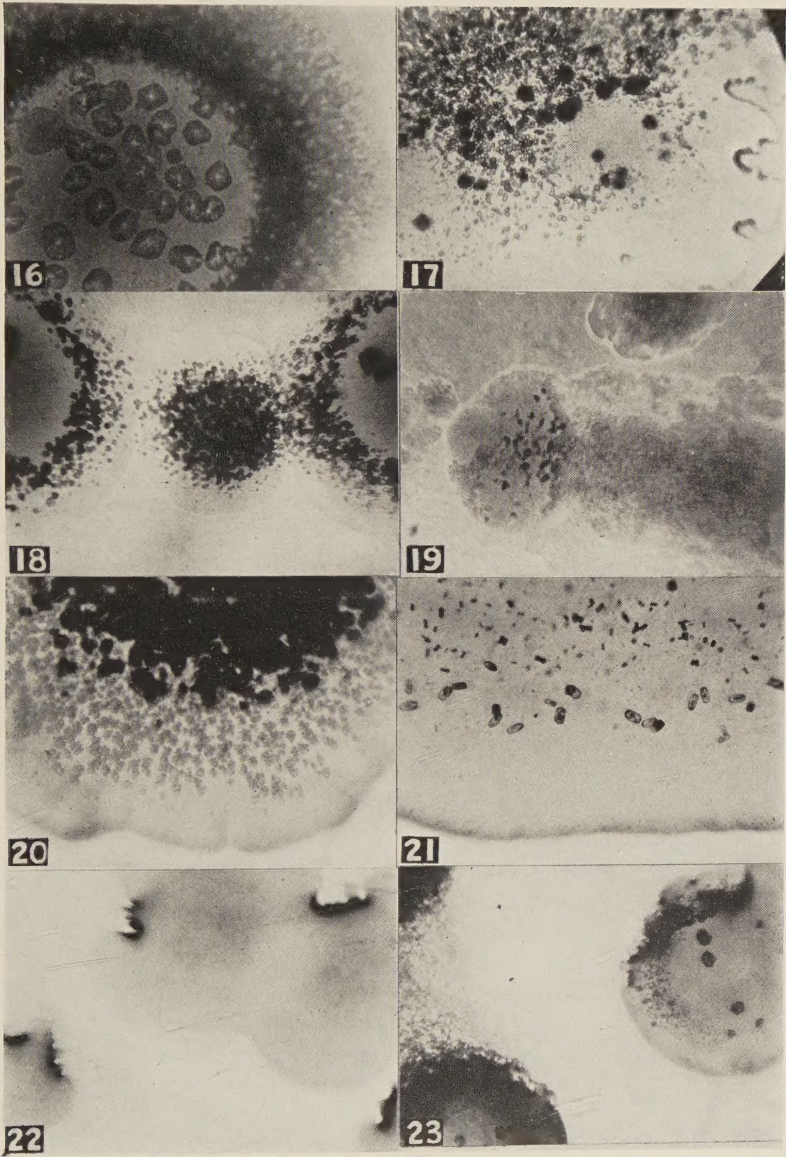
FIG. 19. *E. coli*, m/10 NaCl agar, eighth transfer; twenty-four hours 37°C . $\times 40$.

FIG. 20. *E. coli*, m/5 CaCl_2 agar, eleventh transfer; seven days 37°C . $\times 40$.

FIG. 21. *S. Schottmülleri*, m/10 NaCl agar, thirteenth transfer; forty-eight hours 37°C . $\times 40$.

FIG. 22. *S. Schottmülleri*, m/10 NaCl agar, thirteenth transfer; forty-eight hours 37°C . $\times 40$.

FIG. 23. *E. typhi*, m/5 CaCl_2 agar, fifteenth transfer; forty-eight hours 37°C . $\times 40$.



(Mary E. Caldwell: Dissociation of Paratyphoid Bacilli)

